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(54) Title: SYNTHESIS OF HYALURONIC ACID

(57) Abstract

A preparative enzymatic synthesis of hyaluronic acid (HA) from UDP-N-acetyl-D-glucosamine (UDP-GlcNAc) and UDP-glucuronic acid (UDP-GlcA) catalyzed by hyaluronic acid synthase is coupled with regeneration of the sugar nucleotides. Polymerizing UDP-GlcA and UDP-GlcNAc to form hyaluronic acid results in the formation of released UDP. The released UDP is, in turn, employed in the regeneration of UDP-GlcA and UDP-GlcNAc. Use of the released UDP for regenerating UDP-GlcA and UDP-GlcNAc prevents a build-up of these compounds and prevents or reduces feed back inhibition of the hyaluronic acid synthase reaction that would otherwise be caused by such build-up. Accordingly, the product yield is enhanced by the recycling of these compounds.

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Synthesis of Hyaluronic Acid

Description

Technical Field

The invention relates to the enzymatic synthesis of hyaluronic acid. More particularly, the invention relates to the enzymatic synthesis of hylauronic acid with the regeneration of sugar nucleotides.

Background of the Invention

Hyaluronic acid (HA) is a linear high molecular weight (>5 x 10^6 Da) glycosaminoglycan composed of β-1,4-linked repeating disaccharide units of GlcA β-1,3-linked to GlcNAc. (A. Markovitz et al., *J. Biol. Chem.* (1959): vol. 234, p 2343; K. Sugahara et al., *J. Biol. Chem.* (1979): vol. 254, p 6252; and P. Prehm, *Biochem. J.* (1983): vol. 211, p 191.) It possesses unique viscoelastic and rheological properties. (E.S. Rosen, Viscoelastic Materials: Basic Sciences and Clinical Applications, Pergamon: New York, 1989; S.M.A. Holmbeck et al., *Biochemistry* (1994): vol. 33, p 14246.) Hyaluronic acid is also involved in many important biological processes. For example, the role of hyaluronic acid with respect to hemopoiesis is characterized by M. Siczkowski et al. (*Exp. Hematol.* (1993): Vol. 21, p 126.) The role of hyaluronic acid with respect to angiogenesis is characterized by J.J.G. Brown et al. (*Differentiation* (1992): vol. 52, p 61.) The role of hyaluronic acid with respect to cell adhesion is characterized by B.P. Toole et al. (*Curr. Opin. Cell Biol.* (1990): vol. 2, p 839) and by C. Hardwick et al. (*J. Cell. Biol.* (1992): vol. 117, p 1343). Hyaluronic acid has been used clinically for viscosupplementation in

ophthalmic surgery. (K.L. Goa, K.L. et al., *Drugs* (1994): vol. 47, p 536.) Hyaluronic acid has been used clinically for treatment of osteoarthritis.

Due to its importance in biomedicine, various modifications of hyaluronic acid have been undertaken to improve its biological properties, e.g., see T. Pouyani et al., *J. Am. Chem. Soc.* (1994): vol. **116**, p 7515. Hyaluronic acid was initially obtained by extraction from rooster comb or umbilical cord. However, mucoid streptococcal bacteria subsequently became a more reliable source. (J. van Brunt, *Bio/Technology* (1986): vol. **4**,p 780; and M. O'Regan, et al., *Int. J. Biol. Macromol.* (1994): vol. **16**(6), p 283.) However, viral contamination is viewed as a potential problem of this bacterial source of hyaluronic acid. Furthermore, excessive dispersed molecular weights are often encountered in these preparations. Development of enzymatic synthesis of hyaluronic acid may provide an alternative source of this important biopolymer and offers opportunities for the preparation of low molecular weight hyaluronic acid and analogs as hyaluronic acid receptors recognize short hyaluronic acid. (C. Underhill, *J. Cell. Sci.* (1992): vol. **103**, p 293.)

The biosynthesis of hyaluronic acid has been studied but not well understood. It was not clear whether the polymerization process is primer dependent until the recombinant hyaluronic acid synthase from Streptococcus pyogenes was expressed in E. coli and shown to catalyze the synthesis of hyaluronic acid from UDP-GlcNAc and UDP-GlcA in a radiolabeled assay. (P.L. DeAngelis et al., *Biochemistry* (1994): vol. **33**, p 9033.) For another work on cloning and sequencing of the HA gene, see M. Lansing, et al. *Biochem. J.* (1993): vol. **289**, p 179.

The enzymatic synthesis of hyaluronic acid using hyaluronic synthase is generally inefficient and provides poor yields.

What is needed is an method for enzymatically synthesizing hyaluronic acid using hyaluronic acid synthase in a more efficient fashion to provide higher yields.

Summary of the Invention:

The invention is directed to a preparative enzymatic synthesis of hyaluronic acid (HA) from UDP-N-acetyl-D-glucosamine (UDP-GlcNAc) and UDP-glucuronic acid (UDP-GlcA) catalyzed by hyaluronic acid synthase coupled with regeneration of the sugar nucleotides (Figure 1).

More particularly, the invention is directed to an improved method for enzymatically synthesizing hyaluronic acid using hyaluronic acid synthase for polymerizing UDP-GlcA and UDP-GlcNAc while simultaneously regenerating the UDP-GlcA and the UDP-GlcNAc consumed during such polymerization. Polymerizing UDP-GlcA and UDP-GlcNAc to form hyaluronic acid results in the formation of released UDP. The released UDP is, in turn, employed in the regeneration of UDP-GlcA and UDP-GlcNAc. Use of the released UDP for regenrating UDP-GlcA and UDP-GlcNAc prevents a build-up of these compounds and prevents or reduces feed back inhibition of the hyaluronic acid synthase reaction that would otherwise be caused by such build-up. Accordingly, the product yield is enhanced by the recycling of these compounds.

Regenerataion of UDP-GlcA is achieved by five substeps.

1. The released UDP is converted to UTP by addition of phosphoenol pyruvate and pyruvate kinase. This reaction results in the formation of both UTP and pyruate.
2. UDP-Glc is formed from the UTP of the first substep by addition of UDP-Glc pyrophos-phorylase. This reaction results in the formation of both UDP-Glc and pyrophosphate.

3. The pyrophosphate of substeps 2 above 6 below is eliminated by by addition of inorganic pyrophosphatase.
4. UDP-GlcA is regenerated from the UDP-Glc formed in substep 3 by addition of NAD and UDP-GlcA dehyrogenase. This reaction results in the formation of both UDP-GlcA and NADH.
5. The NAD consumed in substep 4 is regenerated from the NADH formed in substep 4 by addition of lactate dehyrogenase. This reaction consumes the pyruate formed in substep 1 and results in the formation of both NAD and lactate.

Regeneration of UDP-GlcNAc is achieved by the following additional substep:

6. The UDP-GlcNAC is regenerated from the UTP formed in said Substep 1 by addition of GlcNAc-1-P and UDP-GlcNAc pyrophosphorylase. This reaction results in the formation of both UDP-GlcNac and pyrophosphate.

Brief Description of Drawings:

Figure 1 illustrates the enzymatic synthesis of hyaluronic acid with concomitant regeneration of sugar nucleotides.

Figure 2 illustrates the strategy for the cloning of UDP-GlcNAc pyrophosphorilase.

Figure 3 illustrates the strategy for the cloning of UDP-Glc Dehydrogenase.

Figure 4 illustrates the influence of IPTG concentration (vertical), induction OD_{600} = 0.5, and

temperature (bottom chart: post induction at 22 ° C; top chart: post induction at 30 °C) on the productivity (horizontal) of UDP-GlcNAc Pyrophosphorilase.

Figure 5 illustrates the influence of IPTG concentration (vertical), induction OD_{600} = 0.5 (top and middle charts), induction OD_{600} = 0.5 (bottom chart) and temperature (bottom and middle chart: post induction at 22 ° C; top chart: post induction at 30 °C) on the productivity (horizontal) of UDP-Glc Dehydrogenase.

Figure 6 illustrates an SDS-PAGE analysis of UDP-Glc Dehydrogenase (top figure) and UDP-GlcNAc Pyrophosphorilase (bottom figure). Lane A represents the molecular weight markers which were used in the analysis. Lane B represents the crude extract before IPTG induction. Lane C represents the crude extract after 4 hours for UDP-Glc Dehydrogenase or 6 hours for UDP-GlcNAc Pyrophosphorilase from the induction. Lane D represents the pure enzyme which was purified by chelation affinity chromatography.

Figure 7 illustrates the restoration of activity, Units/mL (vertical axis), in two UDP-Glc Dehydrogenase fractions (Fr.1 and Fr. 2) which were eluted from the Ni^{2+}NTA column by the addition of 1 mM UDP-Glc and 1 mM ß-mercaptoethanol and followed over a period of 1-5 days (horizontal axis).

Figure 8 illustrates the analysis of the stability of UDP-Glc Dehydrogenase which was immobilized on an immobilized on Ni^{2+}NTA resin. The stability is represented by Units/mL of resin (vertical) with a period of 1-6 days (horizontal).

Figures 9A and 9B illustrates enzyme stability as represented by remanent percentage activity (vertical) of UDP-Glc Dehydrogenase (top chart) and UDP-GlcNAc Pyrophosphorilase (bottom chart) over a period of 24 hours (horizontal) at 25 °C .

Figures 10A and 10B illustrates the effect of pH (horizontal axis) on the enzymatic activity (vertical axis) of UDP-Glc Dehydrogenase (top chart) and UDP-GlcNAc Pyrophosphorilase (bottom chart) with 3 different buffers: phosphate buffer (diamond), HEPES buffer (square) or tris/HCl buffer (triangle).

Figures 11A and 11B illustrates the effect of UTP concentrations (horizontal; represented as UTP or 1/UTP) on UDP-GlcNAc Pyrophosphorilase activity (vertical; represented as V or 1/V).

Figures 12A and 12B illustrates the effect of GlcNAc-1-Phosphate concentrations (horizontal; represented as GlcNAc-1P or 1/GlcNAc-1P) on UDP-GlcNAc Pyrophosphorilase activity (vertical; represented as V or 1/V) and (bottom).

Figures 13A and 13B illustrates the effect of UDP-Glc concentrations (horizontal; represented as UDPG or 1/UDPG) on UDP-Glc Dehydrogenase activity (vertical; represented as V or 1/V).

Figures 14A and 14B illustrates the effect of NAD concentrations (horizontal; represented as NAD or 1/NAD) on UDP-Glc Dehydrogenase activity (vertical; represented as V or 1/V).

Detailed Description of the Invention:

Hyaluronic acid with a molecular weight of ~5.5 x 10^5 has been prepared in >30 mg quantities from UDP-N-acetyl-D-glucosamine (UDP-GlcNAc) and UDP-D-glucuronic acid (UDP-GlcA) using hyaluronic acid synthase coupled with regeneration of the sugar nucleotides. Two key enzymes used in the cofactor regeneration, i.e. UDP-GlcNAc pyrophosphorylase and UDP-glucose dehydrogenase, have been overexpressed in *E. coli*. This procedure demonstrates the utility of sugar nucleotide regeneration in the enzymatic synthesis of high molecular weight polysaccharides.

Synthetic Methods

1. General

All chemicals were purchased from commercial sources as reagent grade. UV-visible spectra were recorded on a Beckman DU-70 spectrometer. SDS-PAGE was performed on a Pharmacia Phast-System. Fast protein liquid chromatography was carried out on a Pharmacia system composed of two P-500 pumps, a GP-250 gradient programmer, and a single-path UV-1 monitor. HPLC analysis was performed on a Gilson Gradient HPLC system composed of two 302 pumps, a 811 mixer, a 802B manometric module and a UV detector. Ultracentrifugation was performed on a Beckman L8-80M. NMR spectra were recorded on a Bruker AMX-500 spectrometer. Multi angle laser light scattering (MALLS) was carried out on a Wyatt Dawn DSp-F photometer coupled with a GPC-HPLC system (column Shodex B-803 and B 806) and RI (Erna). A Beckman liquid scintillation system LS-3801 was used for the radiochemical assays. UDP-Glc

dehydrogenase, DNAse and Proteinase-K were purchased from Boehringer Mannheim (Mannheim, FRG). UDP-GlcA (^{14}C), 25 μCi/mL, 251 mCi/mmol, was purchased from ICN (Irvine, CA). Glc-1-P (^{14}C), 10 μCi/mL, 267 mCi/mmol was purchased from Moravek Biochemicals (Brea, CA). 5-6 3H-UTP (38 Ci/mmol) was from ICN. *Streptococcus equisimilis* D181 was from Fidia Advanced Biopolymers and was grown at 37 °C in Brain Heart Infusion Medium (Difco Laboratories, Detroit, MI) with 0.5% glucose (Gibco Laboratories, Grand Island, N.Y.). E. coli K12 (ATCC 10798) and E. coli K5 (ATCC 23508) were obtained from American Type Culture Collection The vector pTrcHis was obtained from Invitrogen Co. (San Diego, CA). The host strain XL1-Blue MRF' was purchased from Stratagene Co. (San Diego, CA). The microorganisms were maintained on LB (Luria-Bertani) medium. When host strains harbored with plasmids, LB medium containing 250 μg/mL of ampicillin was used. Unless otherwise indicated, all the other enzymes and reagents were from Sigma (St. Louis, MO).

2. Preparation of crude membrane-bound HA synthase

To examine the feasibility of this enzymatic reaction for the synthesis of hyaluronic acid (HA) on large scales, we prepared the crude membrane-bound HA synthase from *Streptococcus equisimilis* strain D181 and tested its synthetic activity (synthase obtained from Fidia Advanced Biopolymers, Italy; the membrane-bound HA synthase was isolated according to the procedure described by Prehm et. al. *Biochem. J.* **1986**, *235*, 887).

a) Extraction of streptococcal membrane fraction

The isolation of the streptococcal membrane fraction was carried out according to the procedue from Prehm et. al. *Biochem. J.* **1986**, *235*, 887, with

some modifications as follows. A streptococcal culture was treated in the mid-log phase (OD_{600} = 0.5) for 30 min at 37 °C with 6000 units of hyaluronidase/liter and subsequently harvested by centrifugation (6000 x g , 10 min, 4 °C). The bacteria were washed three times with a solution of ice-cold PBS (phosphate buffer saline), pH 7.4, containing the following protease inhibitors: Benzamidine (1 mM), Apoprotinine (2 μg/mL), Pepstatine (1 μg/mL), and Antipaine (1 μg/mL) and finally resuspended in 10 mL of the same solution. The cells were disrupted by sonication for 2 min (repeated for five times) at 120 Watt and treated with DNAse, 10 μg/mL, for 15 min. The bacterial debris were removed by centrifugation (7500 x g, 10 min, 4 °C). The membrane fraction was collected by ultracentrifugation (120,000 x g, 45 min, 4 °C), resuspended in 100 mM HEPES buffer, pH 7.5, and immediately frozen in liquid nitrogen and stored at -70 °C.

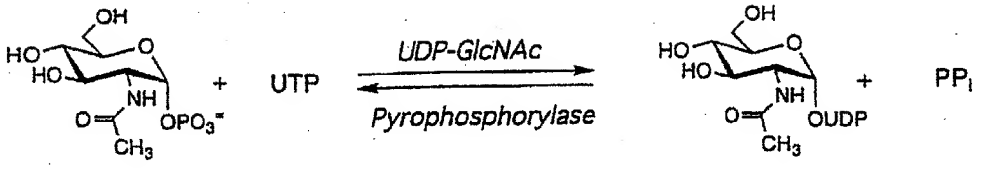
b) HA synthase activity assay

Coupled Enzymatic Test. The initial reaction rate of the HA-synthase was determined using a modified continous coupled spectrophotometric assay method as shown in Figure 1. Fitzgerald, D.K.; Colvin, B.; Mawal, R.; Ebner, K.E. *Anal. Biochem.* **1970**, *36*, 43; Palcic, M.M.; Hindsgaul, O. *Glycobiology* **1991**, *1*, 205. Reactions were carried out at 25 °C in 1 mL plastic cuvettes containing 2 mM phosphoenolpyruvate, 0,25 mM NADH, 50 mM KCl, 10 mM $MgCl_2$, 25 U lactate dehydrogenase, 25 U pyruvate kinase, 2 mM UDP-GlcA, 2 mM UDP-GlcNAc, 40 mM NaCl, 4 mM DTT, 100 mM HEPES, pH 7.5 and 50 μg plasma membrane proteins/mL. The formation of UDP was followed by monitoring the decrease in absorbance of

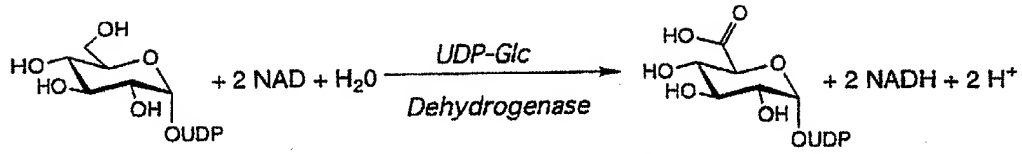
NADH at 340 nm. The background activity was calculated by incubating an aliquot of the same membrane fraction preparation under the same condition in the absence of the UDP-sugars. The reaction rates and specific activities of the membrane preparations were determined based on the extinction coefficient of 6.22 mM^{-1} cm^{-1} for NADH. The protein content was estimated using the BCA protein assay kit (Pierce). The activity was calculated assuming that the addition of 1 μmol of sugar-precursor per min to the growing hyaluronate chain is one unit of hyaluronate synthase activity. This is equivalent to one μmol of NAD produced per minute.

c) Radiochemical assay

Samples of Streptococcal membrane fractions (100 μL) to be tested for hyaluronate synthase activity were incubated in 100 mM HEPES, pH 7.5, with 1 mM, 5 mM or 10 mM UDP-GlcA, 1 mM, 5 mM or 10 mM UDP-GlcNAc, 10 mM $MgCl_2$ and 4 mM DTT in 1 mL. To the sample, 0.1 μCi of UDP-GlcA (^{14}C) was added (before the addition of the membrane). The reactions were incubated at 25 °C with gentle shaking and aliquots were taken at different time intervals. The reactions were stopped by adding SDS to a final concentration of 0.1% and boiling the sample for three minutes. Every sample was divided into two equal aliquots; the first aliquot was loaded onto a silica-gel plate for chromatography; autoradiography of the plates allowed for the detection of the spots corresponding to the polymers and the unreacted UDP-GlcA. The spots were scraped from the plate and the radioactivity in cpm was counted with the liquid scintillation counter. The other aliquot was counted directly as control.



Scheme I



Scheme II

d) Enzyme Stability Study

The membrane fraction was incubated at 25 °C and at 37 °C in HEPES, pH 7.5. At different time intervals, aliquots were taken and assayed for the HA synthase activity with the coupled enzymatic reactions as described above.

3. Regeneration of sugar nucleotides

The enzyme preparation was indeed found to catalyze the synthesis of HA from UDP-GlcNAc and UDP-GlcA, though the yield was only around 20%. In order to improve the yield and to scale up the process, both sugar nucleotides were then regenerated *in situ* from UDP as shown in Scheme I. It was shown that regeneration of sugar nucleotides in glycosyltransferase reactions would reduce the cost of sugar nucleotides and the problem of product inhibition, and make the enzymatic synthesis practical for large scale process. Wong et. al. *J. Org. Chem.* **1992**, *47*, 5416; Wong et. al. *J. Am. Chem. Soc.* **1991**, *113*, 4698; Wong et. al. *J. Am. Chem. Soc.* **1992**, *114*, 9238.

4. Preparation of UDP-GlcNAc pyrophosphorylase

The enzymes required for the cofactor regenerations are commercially available except UDP-GlcNAc pyrophosphorylase (EC 2.7.7.23) which has been overexpressed in *E. coli* in this study. The *E. coli* gene *glmU* coding from the enzyme was amplified by PCR using the 5'-primer ATATTGGATCCTTGAATAATGCTATG and the 3'-primer GCGCGAATTCTTACTTTTTCTTACCGGACG digested with BamHI and EcoRI inserted to pTrc-His-A vector and transformed into supercompetent epicurean *E. coli* XL1 blue MRF's cells for overexpression of the enzyme (300 U/L).

a) Cloning of UDP-GlcNAc Pyrophosphorylase (EC 2.7.7.23) Gene

Amplification of the Gene. PCR amplification was performed in a 100 μL reaction mixture containing 1 μL (1.5 μg) of *E. coli* K12 DNA, 300 nmoles of primers glmU-5 and glmU-3 (Scheme I), 200 mM of different dNTPs, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, and 2 units of *Thermus aquaticus* DNA polymerase. Walker, J.E. et. al. *Biochem. J.* **1984**, *224*, 799; Mengin-Lecreulx et. al. *J. Bact.* **1993**, *175* (19), 6150. The mixture was overlaid with mineral oil and subjected to 35 cycles of amplifications. The cycle conditions were set as follows: denaturation, 94 °C for 2 min, 94 °C for 1 min, 55 °C for 2 min; and elongation, 72 °C for 1.5 min.

b) Construction of a UDP-GlcNAc pyrophosphorylase expression vector.

The DNA obtained from PCR amplification was extracted with phenol/chloroform and precipitated with ethanol at -70 °C for 30 min. The DNA was dissolved in a restriction enzyme buffer (A buffer) supplied by Boehringer Mannheim Biochemical Co. (Indianapolis, IN) and digested with BamH-I and EcoR-I at 37 °C for 2 h. The digested DNA was then recovered by phenol/chloroform extraction and ethanol precipitation (70% of final ethanol concentration containing 10% of 3N Na-acetate, pH 5.2), and purified by agarose (0.8%) gel electrophoresis. The DNA band corresponding to 1370 bp size was isolated from the agarose gel, extracted with QIAEX gel extraction kit (Qiagen Co., Chatworth, CA) and eluted with TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.5). This DNA was used as insert. The vector pTrc-His-A was also digested with 5 U/mg DNA of BamH-I and

EcoR-I and recovered by ethanol precipitation after the extraction with phenol/chloroform. The restriction enzyme-digested vector was further purified on agarose gel as described above. The insert was then ligated with the restriction enzyme-cutted vector with T4 DNA ligase. Maniatis, T.; Fritsh, E.F.; Sambrook, *J. Molecular cloning: A Laboratory Manual*; Cold Spring Harbor, New York **1989**. The ligated DNA was transformed into supercompetent epicurean *E. coli* XL1-Blue MRF strain and plated on LB agar plates which contained 250 μg/mL ampicillin.

c) Screening for positive clones and expression of the targeted protein

The PCR method was used in screening for the positive clones. Since the *E. coli* XL-1 Blue host strain also contains a similar gene, there may have some background amplification for non-recombinants. However, the positive clones showed very intensive amplification which formed a dense band on agarose gel (0.8%) due to the higher copy number of the target gene present in the cells. Twenty colonies were randomly selected from plates and lysed with 50 mL of cell lysing buffer (20 mM Tris-HCl containing 1% Triton X-100 and 2 mM EDTA, pH 8.5). Heated with boiling water for 5 min, the solution was used directly as a DNA template source for PCR amplification. The procedure for the PCR amplification was the same as that described for the amplification of this gene except that 3 μL of the cell lysing solution was used to replace *E. coli* DNA. The colonies which gave intensive PCR amplification were further grown on LB medium containing 250 μg/mL ampicillin and then the plasmids were extracted. The isolated plasmids were further used as template for another PCR reaction, and the product analyzed on

agarose gel to confirm the UDP-GlcNAc-pyrophosphorylase gene insert. The positive clones were selected and used for protein expression.

d) Growing transformed E. coli strain

The transformed *E. coli* strain was grown on LB medium containing 250 μg/mL of ampicillin to mid logarithmic phase (OD_{600} 0.4-0.5) at 37 °C and then induced with 250 μM of IPTG. After the induction the temperature was reduced to 30 °C for the bacteria to grow for another 8 h. Typically one liter of culture would produce ~300 U of the enzyme. The expression level of the recombinant enzyme was followed with time and examined by SDS-PAGE in a Phastsystem (Pharmacia Co.) using precasted gels with a 10-15% gradient of polyacrilamide. The most productive clone for each enzyme was selected and analysis of the influence of IPTG and of the temperature post-induction was carried out.

e) Purification of the UDP-GlcNAc pyrophosphorylase

A crude extract of the enzyme was obtained from the transformed *E. coli*. Briefly, the harvested and washed cells were repeatedly sonicated for 2 min (6x) with cooling. The resulting suspension was centrifuged for 10 min at 200,000 x g. The supernatant fraction was collected and concentrated using Centripep-10 concentrators tubes (Amicon, MA). About 150 mg of protein were loaded onto an anionic exchange column (DEAE-sepharose CL-6B) and eluted with a gradient from 0 to 500 mM of NaCl in 0.02 M phosphate buffer, pH 7.0. The fractions with UDP-GlcNAc pyrophosphorylase activity were pooled together and subjected to another chromatographic purification on FPLC with a Mono-Q 10/10 column eluted with a linear gradient of 0-0.5 M NaCl in 0.02

phosphate buffer, pH 7.0. Fractions (2 mL each) were collected, and the desired fractions containing the pyrophosphorylase were dialyzed against 100 mM HEPES buffer and concentrated by using Microcon-10 concentrators (Amicon, MA). SDS-PAGE was carried out on Phast System by using precasted Phast Gels (acrylamide gradient 10-15) with coomassie blue staining. The purified enzyme (Fig. 2) has a specific activity of 14 U/mg.

f) UDP-GlcNAc pyrophosphorylase activity assay

The assay mixture contained 1 mM GlcNAc-1-P, 10 mM UPT, 5 mM $MgCl_2$ and the enzyme in 100 mM HEPES, pH 7.5. The mixture was incubated at 25 °C for 15, 30 and 60 minutes, and the reactions were terminated by addition of acetic acid (10 % of the mixture's volume). The reaction products were separated by HPLC on a Parsital SAX column (Whatman) eluted with a sodium phosphate buffer, 100 mM, pH 3.5. Quantification of the UDP-GlcNAc was determined by the elution pick's area. In another assay method, H_3-UTP was used. 4 μL of reaction mixture was then mixed with 1 μL of a solution 10 mM UDP-GlcNAc and 10 μM UTP, loaded on a TLC silica gel plate (aluminum flexible plate, Whatman), and developed with in isopropanol / H_2O / NH_4-OAc(1N) with ratio 7 / 2 / 1. The spots corresponding to UTP and UDP-GlcNAc were located by UV absorption and cut out of the plate. The radioactivity was then counted by Beckman liquid scintillation system LS-3801. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 μmole of UDP-GlcNAc per minute.

5. Preparation of UDP-glucose dehydrogenase

The enzyme UDP-glucose dehydrogenase (EC 1.1.1.22) used in the regeneration of UDP-GlcA is

commercially available (from Sigma) but the preparation is impure and very expensive. We have therefore developed an overexpression system to produce this enzyme from *E. coli*.. UDP-glucose dehydrogenase was used in the regeneration of UDP-GlcA (Gygax, D.; Spies, P.; Winkler, T.; Pfarr, U. *Tetrahedron* **1991**, *28*, 5119) and in the synthesis of UDP-GlcA (Toone, E.J.; Simon, E.S.; Whitesides, G.M. *J. Org. Chem.* **1991**, *56*, 5603). To overexpress the enzyme, the gene *kfaC* from *E. coli* strain K5 was amplified by PCR using the 5'-primer ATATTGAGCTCTTCGGAACACTAAAAAA and the 3'-primer GCGCAAGCTTTTAGTCACATTTAAACAAATC, digested with SacI and Hind III, inserted into PTrc-His-A vector and transformed into supercompetent epicurean *E. coli* XL1 blue MRF cells for overexpression of the enzyme (40 U/L).

a) Overexpression and Purification of the Uridinediphosphoglucose Dehydrogenase from *Echerichia coli*.

Uridinediphosphoglucose dehydrogenase UDPG-DH, EC 1.1.1.22) catalyzes the NAD-dependent oxidation of UDP-glucose to UDP-glucuronate (equation 1). UDP-glucose dehydrogenase has been purified to homogeneity from *Escherichia coli* strain MC 153 (Schiller, J.G.; Lamy, F.; Frazier, R.; Feingold, D.S. *Biochem. Biophys. Acta* **1976**, *453*, 418), and has been shown to contain two identical subunits (47 kDa each) in contrast to the six-subunit (52 kDa each) enzyme found in bovine liver (Fitzgerald, D.K.; Colvin, B.; Mawal, R.; Ebner, K.E. *Anal. Biochem.* **1970**, *36*, 43; b) Palcic, M.M.; Hindsgaul, O. *Glycobiology* **1991**, *1*, 205). The recently cloned *hasB* gene from *Streptococcus pyogenes* is the only gene that has been demonstrated to encode a UDP-glucose

dehydrogenase (Dougherty, B.A.; van de Rijn, I. *J. Biol. Chem.* **1993**, *268* (10), 7118). This gene, together with the other two, resides in a contiguous stretch of the 3.2 kilobase-pair streptococcal DNA that seems able to direct hyaluronate biosynthesis (DeAngelis, P.L.; Papaconstantinou, J.; Weigel, P.H. *J. Biol. Chem.* **1993**, *268* (20), 14568). Several attempts of cloning and overexpression of the streptococcal *hasB* gene in *E. coli* have been made, but no active UDP-glucose dehydrogenase has been obtained, presumably due to the difficulty of expressing a gene from a Gram-positive bacteria in Gram-negative *E. coli*. Searching for sequence similarity within the GeneBank using the FASTA program, we found two genes from *E. coli* exhibiting a significant degree of identity with the UDPG-DH from streptococcus: One from strain 0111 (M92) which encodes a "hypothetical protein" of 43.3 kDa (Bastin, D.A.; Stevenson, G.; Brown, P.K.; Haase, A.; Reeves, P. *Mol. Microbiol.* **1993**, *7*(5), 725; The GenBank accession number for the sequence is Z17241). The other, *kfaC*, from *E. coli* strain K5 resides in a contiguous stretch of the 8 kilobase-pair DNA called "region 2 of the K5 antigen gene cluster" (GenBank accession number is X77617) a region that seems involved in the synthesis of the K5 polysaccharide (a polysaccharide very similar to the hyaluronic acid). The streptococcal UDPG-DH and the protein deduced from *E. coli* 0111 gene are 53.5% identical over 402 residues (init = 481), and comparison of the streptococcal protein with the protein deduced from the gene *kfaC* of *E. coli* K5 revealed a 53.8% identity over 400 residues (init = 450). The two genes from *E. coli* are 75.3% identity over 388 residues (init = 1518) (Figure 4). To our knowledge these two genes have not been reported to be responsible for the

dehydrogenase activity.

b) Amplification of the Gene (Figure 3)

PCR reaction was performed in a 100 μL reaction mixture containing 1 μL (1.5 μg) of *E. coli* K5 DNA, 150 nmoles of primers kfaC-5' and kfaC-3', 200 mM of different dNTPs, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, and 2 units of Thermus aquaticus DNA polymerase. The reaction was overlaid with mineral oil and subjected to 35 cycles of amplifications. The cycle conditions were set as follow: denaturation at 94 °C for 1 min, 55 °C for 2 min; and elongation at 72 °C for 1.5 min.

c) Construction of a UDP-Glc dehydrogenase expression vector

The DNA obtained from PCR amplification was extracted with phenol/chloroform, precipated with ethanol (70% of final ethanol concentration containing 10% of 3N Na-acetate, pH 5.2) at -70 °C for 30 min and dissolved in TE buffer (10 mM Tris/HCl and 1 mM EDTA, pH 7.5). An aliquot of the DNA was dissolved in an appropriate restriction enzyme buffer (A buffer) supplied by Boehringer Mannheim Biochemical Co. (Indianapolis, IN) and digested with Sac-I at 37 °C for 2 h. The DNA was recovered by phenol/chloroform extraction and ethanol precipitation, and subsequently dissolved in the restriction enzyme buffer B (Boehringer Mannheim). A second digestion was then performed with Hind-III at 37 °C for 2 h. The double-digested DNA was recovered by another phenol/chloroform extraction and ethanol precipitation, and purified by agarose (0.8%) gel electrophoresis. The DNA band corresponding to the 1180-bp size was isolated from the agarose gel and extracted with QIAEX gel extraction kit (Qiagen Co.,

Chatworth, CA) and eluted with TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.5). This DNA was used as insert. The vector pTrc-His-A was also subjected to a double digestion by Sac-I (in buffer A) and Hind-III (in buffer B), and recovered with ethanol precipitation after extraction by phenol/chloroform. The restriction enzyme-digested vector was further purified on agarose gel as described above. The insert was then ligated with the vector by using T4 DNA ligase (Wierenga, R.K.; Terpstra, P.; Hol, W.G.J. *J. Mol. Biol.* **1986**, *187*, 101). The ligated DNA was transformed into supercompetent epicurean *E. coli* XL1-Blue MRF strain and plated on LB agar plates which contained 250 μg/mL ampicillin.

d) Screening for positive clones and expression of the targeted protein

The PCR method was used in screening for the positive clones. The host *E. coli* XL1-Blue itself does not contain the *kFaC* gene so the colonies showing the amplification must contain the heterologous gene. Ten colonies were randomly selected from plates and grown in 10 mL of LB buffer containing 250 μg/mL of ampicillin. 100 μL of the culture was then taken and centrifuged, and the pellet was resuspended in 50 μL of cell lysing buffer (20 mM Tris-HCl containing 1% Triton X-100 and 2 mM EDTA, pH 8.5). After heating with boiling water for 5 min, the solution was used directly as a DNA template source for PCR amplification. The procedure for the PCR amplification was the same as that described in the amplification of this gene except that 3 μL of the cell lysing solution were used to replace *E. coli* K5 DNA. Three clones which gave the best amplification were selected and investigated for the level of protein expression. The transformed *E.*

coli strains were grown on LB medium containing 250 μg/mL of ampicillin to mid logarithmic phase (OD_{600} 0.4-0.5) at 37 °C and then induced with 50 μM of IPTG. After the induction the temperature was reduced to 30 °C and the bacteria grown for an additional 6 h. The expression level of the recombinant UDP-Glc dehydrogenase was followed with time and examined by SDS-PAGE in a Phast system (Pharmacia Co.) using precasted gels with a 10-15% gradient of polyacrylamide. Typically, one liter of cell culture would produce ~40 U of the enzyme.

e) Purification of the UDP-Glc dehydrogenase A crude extract of the enzyme was obtained from the culture broth of the transformed *E. coli*.. Briefly, the culture was centrifuged and suspended in native-binding buffer (100 mM NaH_2PO_4, 10 mM Tris/HCl, pH 8.0) and disrupted by a French pressure cell and centrifuged at 1500 x g for 30 min. Cells from 250 mL of culture were resuspended in 20 mL of native-binding buffer, the crude extract was prepared and concentrated to 10 mL. The sample was then loaded on a 15 mL column containing 3.5 mL of Ni-NTA resin and equilibrated with the same buffer. The column was washed with native-binding buffer untill no optical density at 280 nm was detectable. The column was then washed with native-binding buffer containing increasing concentration of imidazole (5 mM, 10 mM, 15 mM, 25 mM, and 50 mM). Fractions (1 mL each) were collected and analyzed by SDS-PAGE. The UDP-Glc-DH was eluted from the column when the buffer contained 50 mM of imidazoe (total 5 U, specific activity = 4 U/mg).

Table 1. Summary of purification data for the two enzymes

	Protein (mg)	Total activity (Units)	Specific activity (Units/mg)	Yield (%)	Purification factor
UDP-GlcNAc PP 1 Liter of culture					
Step:					
Cell Free Extract	345	276	0.8	100	1
NiNTA column + conc.	5.5	112	20.4	40.6	25.5
UDP-Glc DH 4 Liters of culture					
Step:					
Cell Free Extract	1200	168	0.14	100	1
NiNTA column + conc.	10.3	70	6.8	41.6	48.6

f) UDP-Glc dehydrogenase activity assay

The UDPG-DH was assayed by following the reduction of NAD at 340 nm at 25 °C in a 1 cm light path cuvette. The reaction mixture contained 1 mM UDP-Glc, 2 mM NAD and 50 mM Tris/HCl pH 8.7. The reaction was initiated by addition of enzyme solution. The initial velocity was estimated during the first minute. A unit of enzyme activityis defined as the amount of enzyme required to produce 2 μmoles of NADH per min.

6. Enzymes stability study

The enzymes were incubated at 25 °C in 100 mM HEPES, pH 8.0, in the presence or absence of one substrate or reducing agent. At different time intervals, aliquots were taken and assayed for the activity. These studies were carried out using pure enzymes.

7. pH dependence

The pH influence on the activity was studied using the pure enzyme. In the case of UDP-GlcNAc PP, to 30 μL of a stock solution containing the enzyme, $MgCl_2$ and ^3H-UTP was added 30 μL of a solution containing GlcNAc-1P and the buffer. The solution was then loaded on a TLC plate as described above. For UDP-Glc DH, to 300 μL of a stock solution containing the enzyme and UDP-Glc was added 300 μL of a solution containing NAD and buffer.

8. Enzymes Kinetics

The influence of the substrate concentration on the initial velocity was measured using pure enzymes and with all other conditions maintaining constant. Also in this case, the radioactivity assay was used

for UDP-GlcNAc PP. The K_m values were calculated by a non linear least square fit to the Michaelis-Menten rate equation curve. A computer program (Hyperl) was used according to Cleland et. al. W.W. *Methods in Enzymology*, 1979, 63, 103.

9. Hyaluronate Identification

Electrophoresis. SDS-PAGE was carried out using the Phast System and precasted gels (4-15%) following the protocol recommended by the manufacturer. Samples of HA from the *in vitro* synthesis were resuspended in a sample buffer (7.8 mM Tris-HCl, 6% w/v urea, 0.875% w/v SDS, 2.5% w/v glycerol, 0.625 mM EDTA, 0.00025% bromophenolblue, pH 8.9). The samples were heated to 100 °C for 3 min. After electrophoresis gels were stained according to the method described by Moller et al. *Anal. Biochem.* 1970, *36*, 43, using a combined alcian blue (Bio-Rad, Richmond, CA) and silver staining procedure. When labeled Glc-1-P(^{14}C) was used in the synthesis of HA, the radioactivity in the pellet was calculated. The pellet was washed twice with 5% trichloroacetic acid by resuspension and centrifugation at 14,000 x g, and then digested in 0.2 M NaOH for 24 hour The cpm of the solution was counted at the counter. The percent of the total Glc-1-P initially present in the reaction solution that resulted incorporated in the pellet was always less than 1%. Formation of hyaluronate was confirmed by digestion with hyaluronate lyase (EC 4.2.2.1), an enzyme that cleaves specifically the ß1-4 linkage between GlcNAc and GlcA yielding 4,5 unsaturated tetra- and exa-saccharides, and by hyalurono-glucoronidase (EC 3.2.1.36).

Table 2. Summary of kinetic constants for the two enzymes

UDP-GlcNAc PP		**UDP-Glc DH**	
Km_{UTP}	12.5 ± 4 µM	$Km_{UDP\text{-}Glc}$	15 ± 2.5 µM
$Km_{GlcNAc1P}$	11.3 ± 1µM	Km_{NAD}	199 ± 20 µM
kcat	2660 min^{-1}	kcat	676 min^{-1}

Gel permeation chromatography. The Hyaluronic acid in the 5% trichloroacetic solution was separated from the unreacted precursors by gel chromatography on a Sepharose CL 4B column (50 cm x 0.65 cm) eluted with phosphate buffer saline, pH 7.4. Fractions of 2.1 mL were collected. When labeled substrates were used in the synthesis, 410 μL of every fraction were mixed with 10 mL scintillation buffer and the radioactivity was determined by a scintillation counter. The fractions corresponding to the excluded volume were pooled (Fig. 3) to recover the HA. This solution was then ultrafiltrated by using centripet-10 (Amicon), digested with hyaluronate lyase, and rerun through the column. The HA peak disappeared, suggesting that the peak at the void volume contains HA.

GPC-MALLS. Determination of the MW of HA was performed by coupling a GPC-HPLC system to a multi angular laser light scattering detector and a refraction index apparatus. The intensity of the scattered light was measured simultaneously by 18 photodiodes and was used to calculate the scattering function for determination of the molecular weight.

10. Prepartive synthesis of Hyaluronic Acid (HA)

With these enzymes available, a preparative synthesis of HA was carried out. In a representative synthesis, to a HEPES (4-(2-hydroxy-ethyl)piperzine-1-ethanesulfonic acid) buffer solution (0.1 M, pH 7.5, total volume = 10 mL) containing Glc-1-P (α-D-Glucose 1-phosphate disodium salt tetrahydrate; 0.1 mmol; commercially available from Fluca), GlcNAc-1-P (α-D-Glucosamine 1-phosphate; 0.1 mmol; commercially available from Sigma), phosphoenol pyruvate (PEP, 0.2 mmol; commercially available from Sigma), NAD (5 μmol; -α-Nicotinamide Adenine Dinucleotide is

commercially available from Sigma), UTP (10 μmol; Uridine 5'-triphosphate is commercially available from Sigma), $MgCl_2 \cdot 6H_2O$ (0.1 mmol), dithiothreitol (40 μmol; Aldrich) and KCl (0.5 mmol) was added recombinant UDP-GlcNAc pyrophosphorylase (10 U; as prepared supra), UDP-Glc pyropho-sphorylase (20 U; commercially available from Sigma), UDP-Glc dehydrogenase (10 U; as prepared supra), pyruvate kinase (200 U; commercially available from Sigma), lactate dehydrogenase (200 U; commercially available from Sigma), inorganic pyrophosphatase (50 U; commercially available from Sigma) and the membrane-bound HA synthase (0.4 U; as prepared supra). The mixture was gently stirred under Argon at 25 °C for 48 hours. The reaction was then stopped by digestion with proteinase-K (500 μg) for 60 min at 37 °C followed by addition of cold trichloroacetic acid to a final concentration of 5% to precipitate proteins. The solution was then centrifuged (14,000 x g, 30 min, 4 °C) and the supernatant was passed through a Sepharose CL-4B column (50 x 0.65 cm) eluted with PBS buffer (0.01 M phosphate, 2.7 mM KCl, 137 mM NaCl). The fractions corresponding to HA were collected (68 mL) and dialyzed against 5-L distilled water (repeated for 5 times, 40 h each) using a dialysis tube with MW cut off of 12,000-14,000 Da, then lyophilized to give 31 mg of HA sodium salt (90% yield). The turnover number for UTP, UDP-GlcNAc and UDP-GlcA was 16. The HA prepared was further characterized by ^{1}H-nmr (D_2O, 500 Hz) and enzymatic digestion by hyaluronate lyase (EC 4.2.2.1; commercially available from Boehringer) and hyalurono glucuronidase (EC 3.2.1.36; commercially available from Boehringer), and the results were the same as that of authentic HA.

Analysis by multiangular laser light scattering

indicates that the average molecular weight of the synthetic HA is ~5.5 x 10^5, corresponding to a degree of polymerization of 1500.

In summary, this study has demonstrated that high molecular weight HA can be synthesized enzymatically from relatively inexpensive substrates: Glc-1-P and GlcNAc-1-P. All the enzymes were quite stable except HA synthase which exhibited a half-life of 24 h at 25 °C.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Wong, Chi-Huey

(ii) TITLE OF INVENTION: SYNTHESIS OF HYALURONIC ACID

(iii) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: The Scripps Research Institute
(B) STREET: 10666 North Torrey Pines Road, TPC-8
(C) CITY: La Jolla
(D) STATE: California
(E) COUNTRY: United States
(F) ZIP: 92037

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT/US95/
(B) FILING DATE: 30-NOV-1995
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Lewis, Donald G.
(B) REGISTRATION NUMBER: 28,636
(C) REFERENCE/DOCKET NUMBER: 497.0 PC

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (619) 554-2937

(B) TELEFAX: (619) 554-6312

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 428 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Phe Gly Thr Leu Lys Ile Thr Val Ser Gly Ala Gly Tyr Val Gly
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Leu Ser Asn Xaa Gly Ile Leu Met Ala Gln Asn His Glu Xaa Xaa Val
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Val Ala Phe Asp Thr His Gln Lys Lys Val Asp Leu Leu Asn Asp Lys
35 40 45

Leu Ser Pro Ile Xaa Glu Asp Lys Glu Ile Glu Asn Tyr Leu Ser Thr
50 55 60

Xaa Xaa Lys Ile Leu Asn Phe Arg Ala Thr Thr Asn Lys Tyr Glu Ala
65 70 75 80

Tyr Lys Asn Ala Asn Tyr Val Ile Ile Ala Xaa Xaa Thr Pro Thr Asn
85 90 95

Xaa Tyr Asp Pro Gly Ser Asn Tyr Phe Asp Thr Ser Ser Val Glu Ala
100 105 110

Val Ile Arg Asp Val Thr Glu Ile Asn Pro Asn Ala Ile Met Val Val
115 120 125

Xaa Xaa Lys Ser Thr Val Pro Val Gly Phe Thr Lys Thr Ile Lys Glu
130 135 140

His Leu Gly Ile Asn Xaa Xaa Xaa Xaa Xaa Asn Ile Ile Phe Ser Xaa
145 150 155 160

Pro Glu Phe Leu Arg Glu Gly Arg Ala Leu Tyr Asp Asn Leu His Pro
165 170 175

Ser Arg Ile Ile Ile Gly Glu Cys Xaa Ser Glu Arg Xaa Xaa Xaa Xaa
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Xaa Xaa Ala Glu Arg Leu Ala Val Leu Phe Gln Glu Gly Ala Ile Lys
195 200 205

Gln Asn Ile Pro Val Leu Phe Thr Asp Ser Thr Glu Ala Glu Ala Ile
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Lys Leu Phe Ser Asn Thr Tyr Leu Ala Met Arg Val Ala Phe Phe Asn
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Glu Leu Asp Ser Tyr Ala Glu Ser Phe Gly Leu Asn Thr Arg Gln Ile
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Asp Ile Ile Ile Thr Asn Arg Met Ser Glu Glu Leu Asn Asp Val Val
405 410 415

Asp Lys Val Tyr Ser Arg Asp Leu Phe Lys Cys Asp
420 425

What is claimed is:

1. An improved method for enzymatically synthesizing hyaluronic acid using hyaluronic acid synthase for polymerizing UDP-GlcA and UDP-GlcNAc with a formation of released UDP, wherein the improvement comprises the following additional step, viz.:

simultaneously regenerating the UDP-GlcA and the UDP-GlcNAc consumed during polymerization using the released UDP,

whereby using the released UDP for regenerating the UDP-GlcA and the UDP-GlcNAc reduces feed back inhibition of the hyaluronic acid synthase by the released UDP and enhances the yield of hyaluronic acid.

2. An improved method for enzymatically synthesizing hyaluronic acid as described in claim 1 wherein:

the UDP-GlcNAc is regenerated using released UDP by simultaneously performing the following substeps:

Substep A: converting the released UDP to UTP and forming pyruvate by addition of phosphoenol pyruvate and pyruvate kinase;

Substep B: regenerating the UDP-GlcNAC from the UTP formed in said Substep A and forming pyrophosphate by addition of GlcNAc-1-P and UDP-GlcNAc pyrophosphorylase; and

Substep C: eliminating the pyrophosphate of said Substep B by addition of inorganic pyrophosphatase.

3. An improved method for enzymatically synthesizing hyaluronic acid as described in claim 1 wherein:

the UDP-GlcA is regenerated using released UDP by simultaneously performing the following substeps:

Substep A: converting the released UDP to UTP and forming pyruate by addition of phosphoenol pyruvate and pyruvate kinase;

Substep B: forming UDP-Glc from the UTP formed in said Substep A and forming pyrophosphate by addition of UDP-Glc pyrophosphorylase;

Substep C: eliminating the pyrophosphate of said Substep B by addition of inorganic pyrophosphatase;

Substep D: regerating the UDP-GlcA from the UDP-Glc formed in said Substep B and forming NADH by addition of NAD and UDP-GlcA dehydrogenase; and

Substep E: regerating the NAD consumed in said Substep D and forming lactate from the NADH formed in said Substep D and the pyruate formed in said Substep A by addition of lactate dehyrogenase.

4. An improved method for enzymatically synthesizing hyaluronic acid as described in claim 1 wherein:

the UDP-GlcA is regenerated using released UDP by simultaneously performing the following substeps:

Substep A: converting the released UDP to UTP and forming pyruate by addition of phosphoenol pyruvate and pyruvate kinase;

Substep B: forming UDP-Glc from the UTP formed in said Substep A and forming pyrophosphate

by addition of UDP-Glc pyrophosphorylase;

Substep C: eliminating the pyrophosphate of said Substep B and of Substep F below by addition of inorganic pyrophosphatase;

Substep D: regerating the UDP-GlcA from the UDP-Glc formed in said Substep B and forming NADH by addition of NAD and UDP-GlcA dehyrogenase; and

Substep E: regerating the NAD consumed in said Substep D and forming lactate from the NADH formed in said Substep D and the pyruate formed in said Substep A by addition of lactate dehyrogenase; and

the UDP-GlcNAc is regenerated using released UDP by simultaneously performing the following additional substep:

Substep F: regenerating the UDP-GlcNAC from the UTP formed in said Substep A and forming pyrophosphate by addition of GlcNAc-1-P and UDP-GlcNAc pyrophosphorylase.

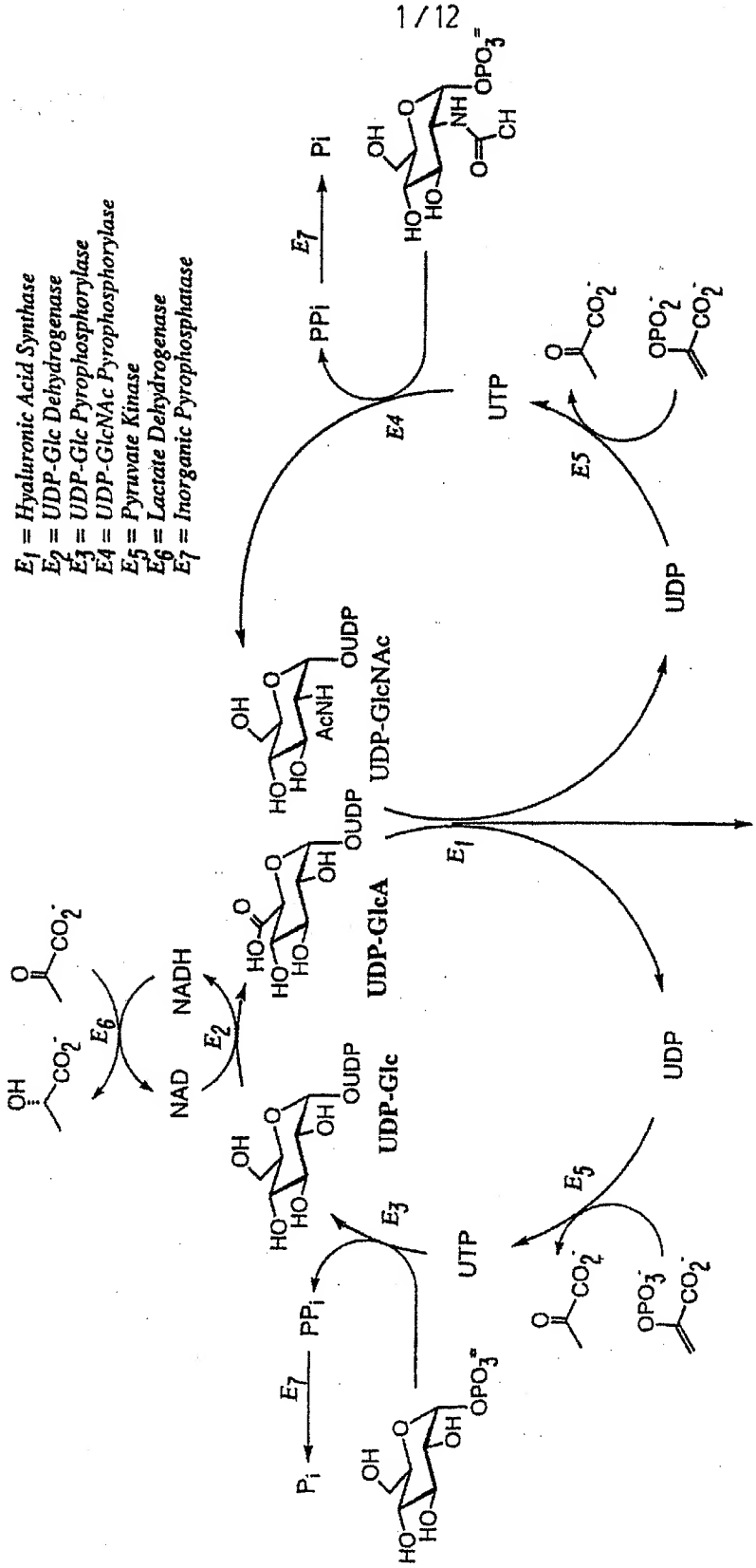
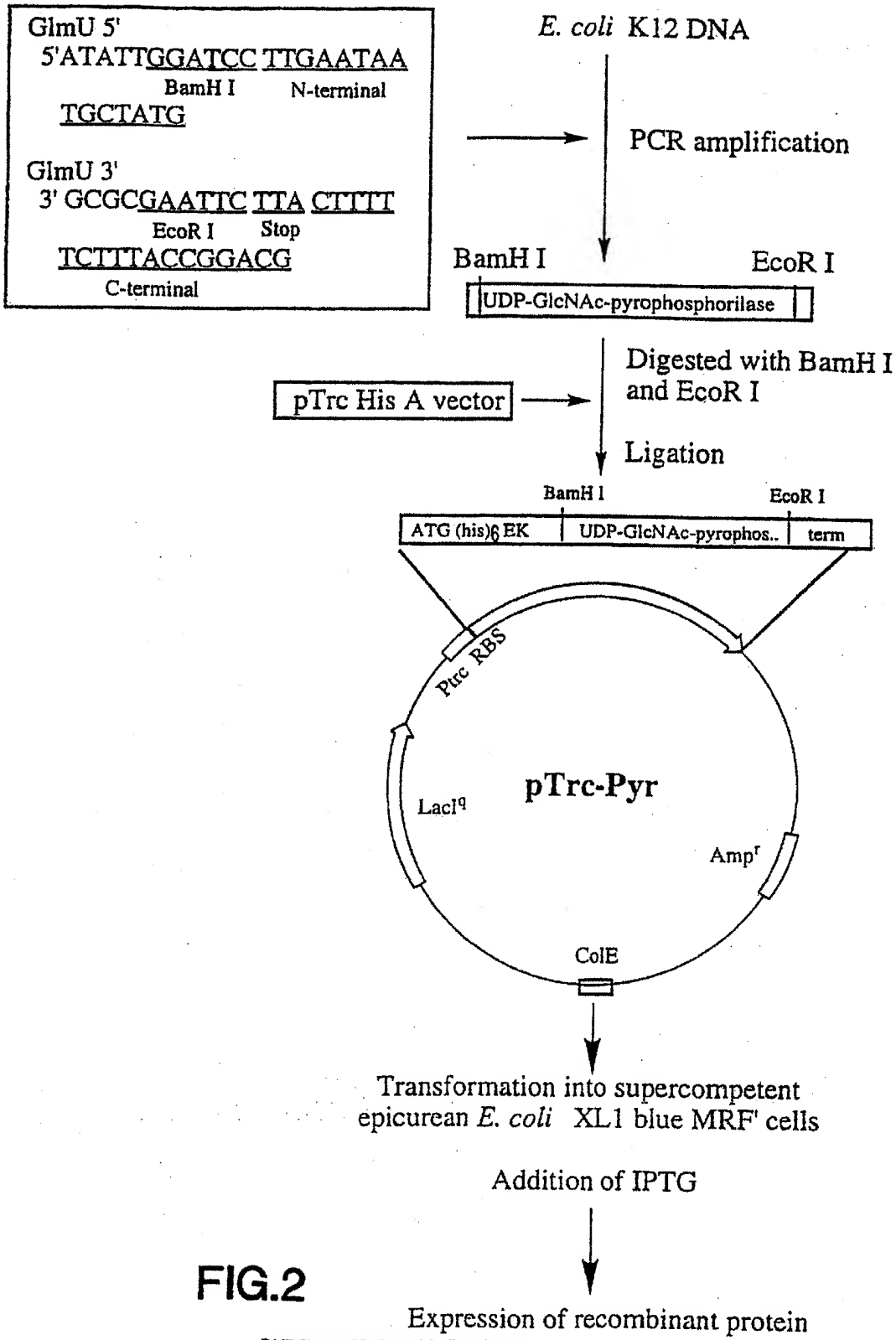

E1 = Hyaluronic Acid Synthase
E2 = UDP-Glc Dehydrogenase
E3 = UDP-Glc Pyrophosphorylase
E4 = UDP-GlcNAc Pyrophosphorylase
E5 = Pyruvate Kinase
E6 = Lactate Dehydrogenase
E7 = Inorganic Pyrophosphatase
UDP-GlcNAc
UDP-GlcA
UDP-Glc
UTP
UDP
NAD
NADH
PPi
Pi
HYALURONIC ACID

FIG.1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BamH I
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TGCTATG
GlmU 3'
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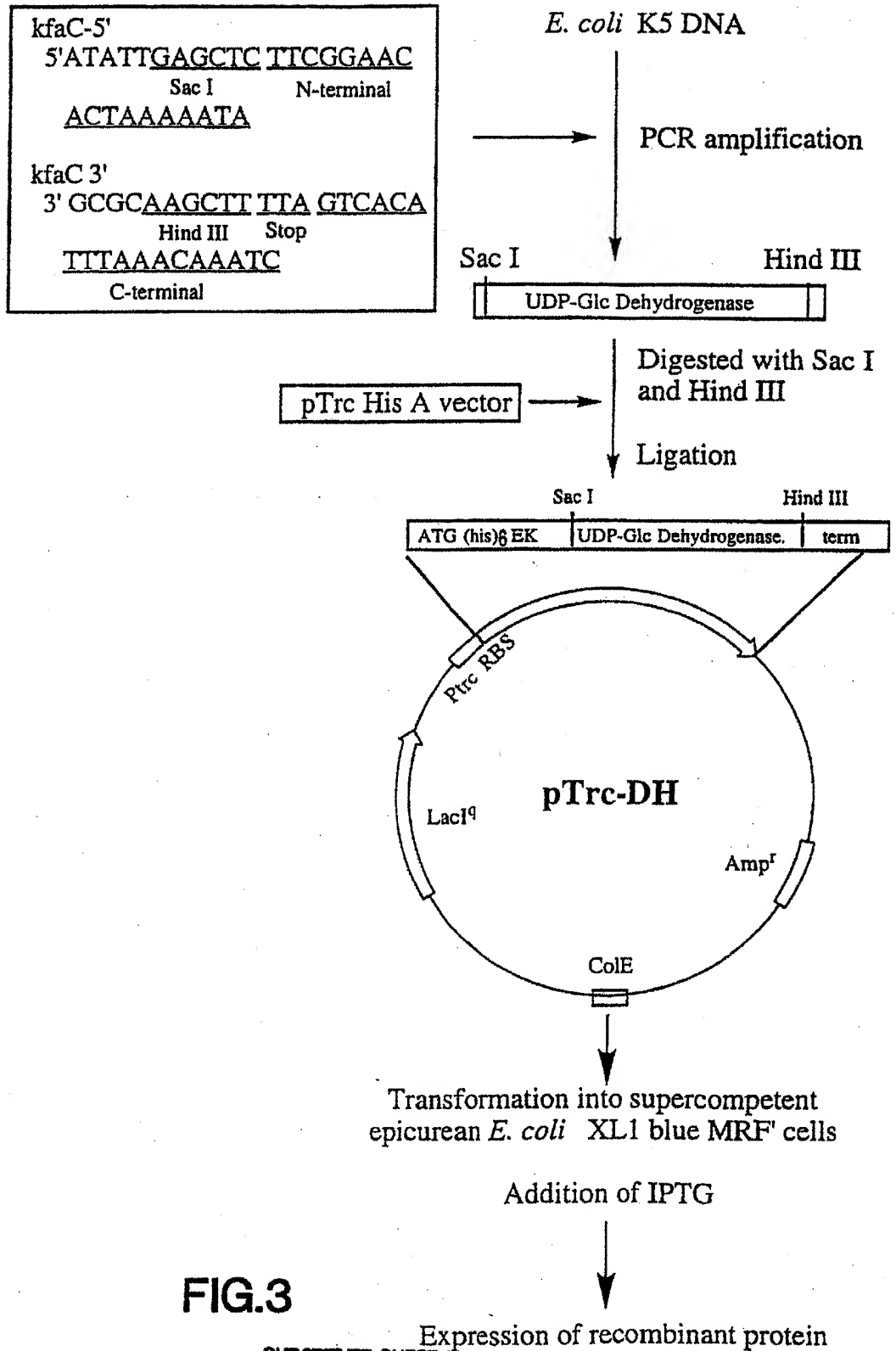
EcoR I
Stop
TCTTTACCGGACG
C-terminal
E. coli K12 DNA
PCR amplification
BamH I
EcoR I
UDP-GlcNAc-pyrophosphorilase
pTrc His A vector
Digested with BamH I and EcoR I
Ligation
BamH I
EcoR I
ATG (his)6 EK
UDP-GlcNAc-pyrophos..
term
Ptrc
RBS
pTrc-Pyr
LacIq
Ampr
ColE
Transformation into supercompetent epicurean E. coli XL1 blue MRF' cells
Addition of IPTG
Expression of recombinant protein

FIG.2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Sac I
N-terminal
ACTAAAAATA
kfaC 3'
3' GCGCAAGCTT TTA GTCACA
Hind III
Stop
TTTAAACAAATC
C-terminal
E. coli K5 DNA
PCR amplification
Sac I
Hind III
UDP-Glc Dehydrogenase
pTrc His A vector
Digested with Sac I and Hind III
Ligation
Sac I
Hind III
ATG (his)6 EK
UDP-Glc Dehydrogenase.
term
Ptrc RBS
pTrc-DH
LacIq
Ampr
ColE
Transformation into supercompetent epicurean E. coli XL1 blue MRF' cells
Addition of IPTG
Expression of recombinant protein

FIG.3

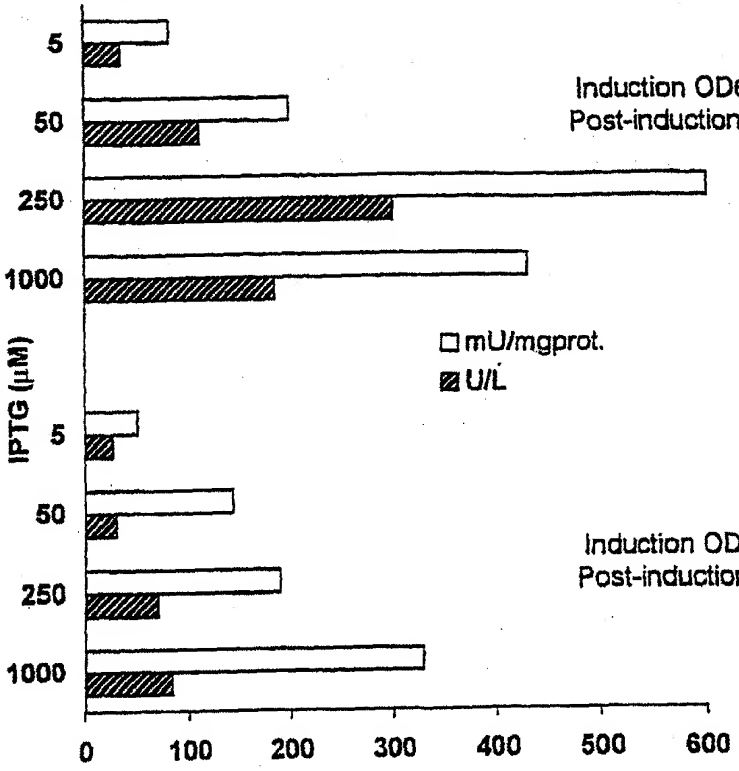
5
50
250
1000
IPTG (µM)
Induction OD600 = 0.5
Post-induction at 30 °C
mU/mgprot.
U/L
5
50
250
1000
Induction OD600 = 0.5
Post-induction at 22 °C
0
100
200
300
400
500
600

FIG.4

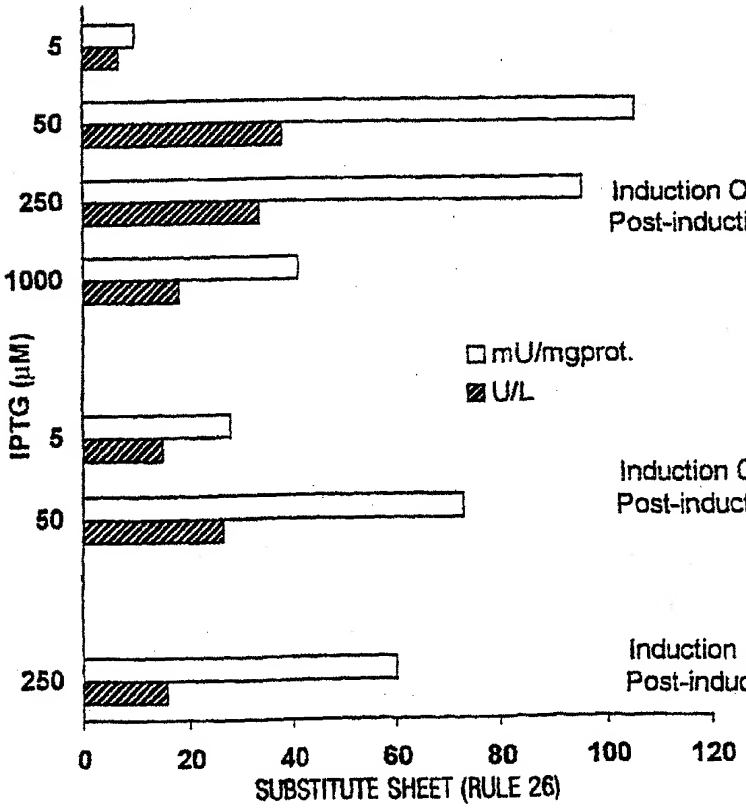
5
50
250
1000
IPTG (µM)
Induction OD600 = 0.5
Post-induction at 30 °C
mU/mgprot.
U/L
5
50
Induction OD600 = 0.5
Post-induction at 22 °C
250
Induction OD600 = 1.0
Post-induction at 30 °C
0
20
40
60
80
100
120

FIG.5

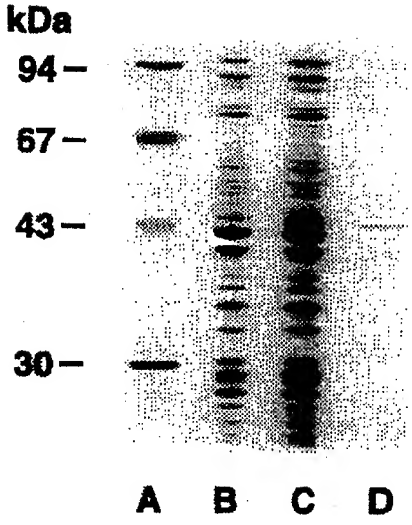

FIG.6A

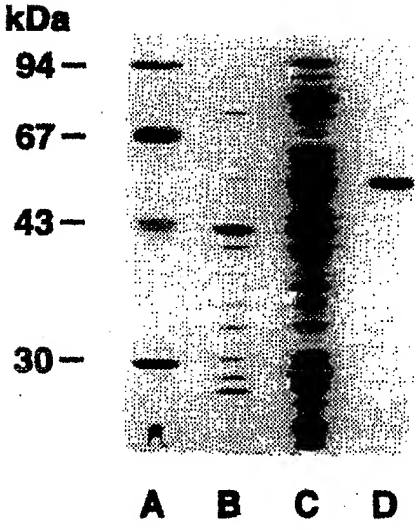

FIG.6B

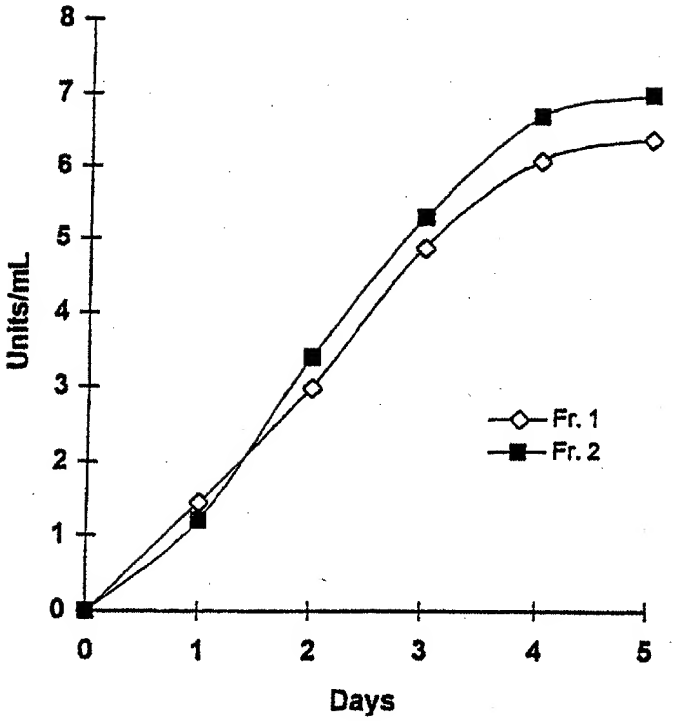
Units/mL
Days
Fr. 1
Fr. 2
0
1
2
3
4
5
6
7
8

FIG.7

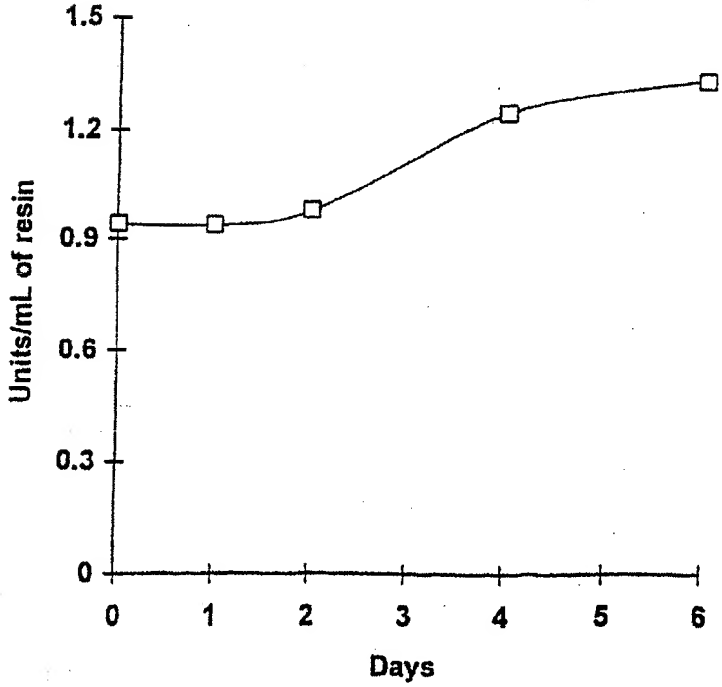
Units/mL of resin
Days
0
0.3
0.6
0.9
1.2
1.5
0
1
2
3
4
5
6

FIG.8

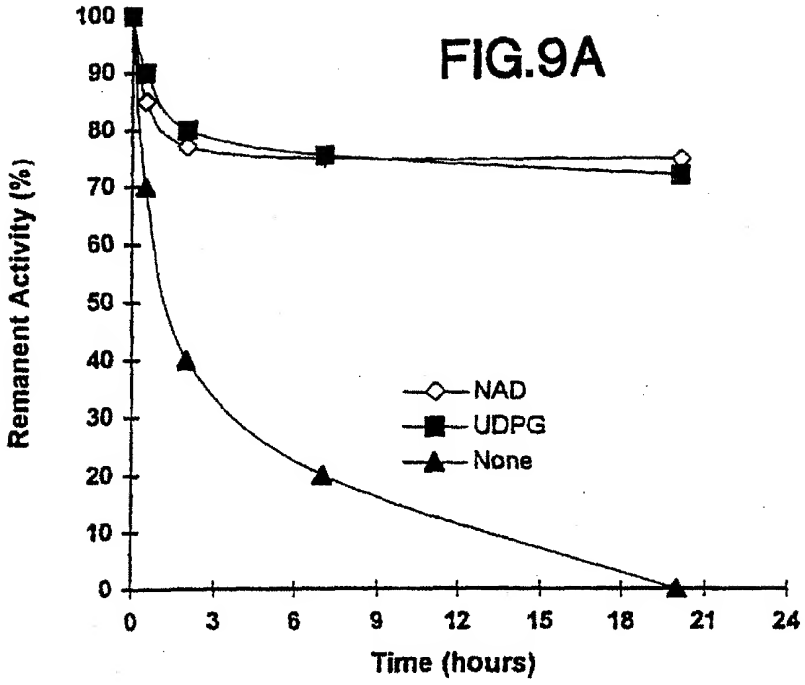
Remanent Activity (%)
100
90
80
70
60
50
40
30
20
10
0
0
3
6
9
12
15
18
21
24
Time (hours)
NAD
UDPG
None

FIG.9A

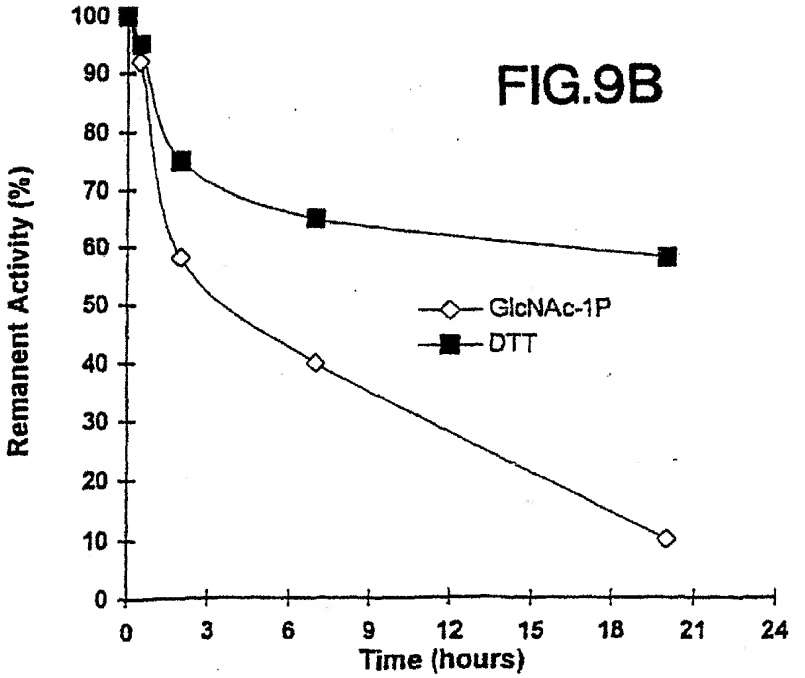
Remanent Activity (%)
100
90
80
70
60
50
40
30
20
10
0
0
3
6
9
12
15
18
21
24
Time (hours)
GlcNAc-1P
DTT

FIG.9B

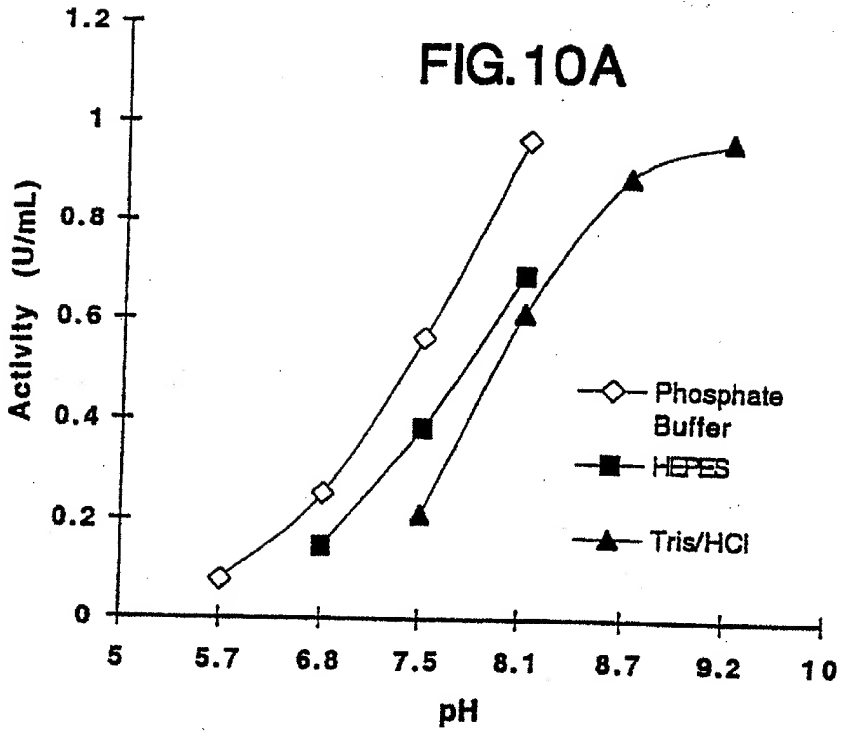

FIG. 10A

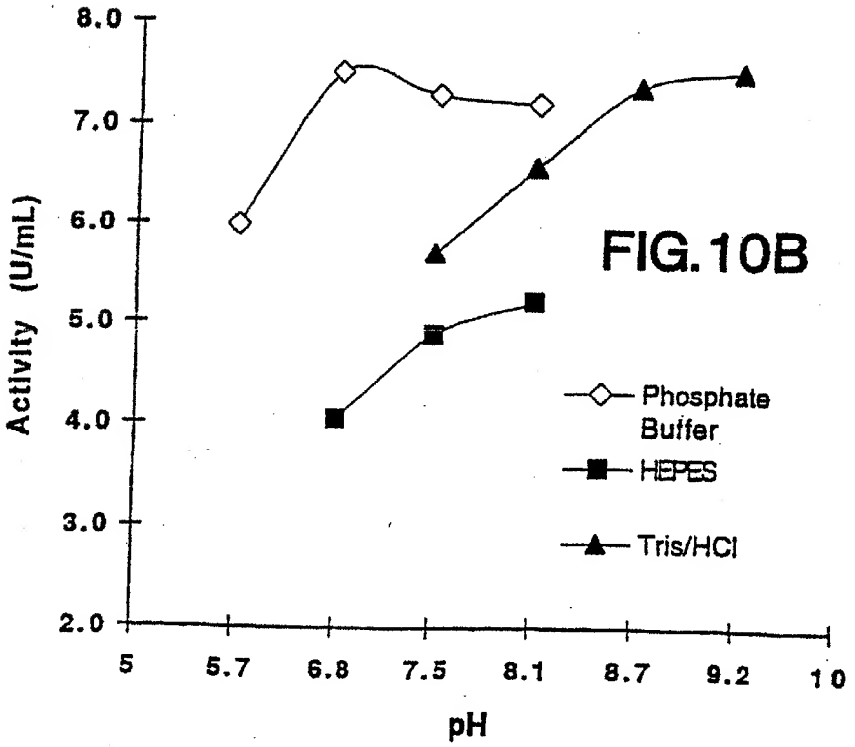

FIG. 10B

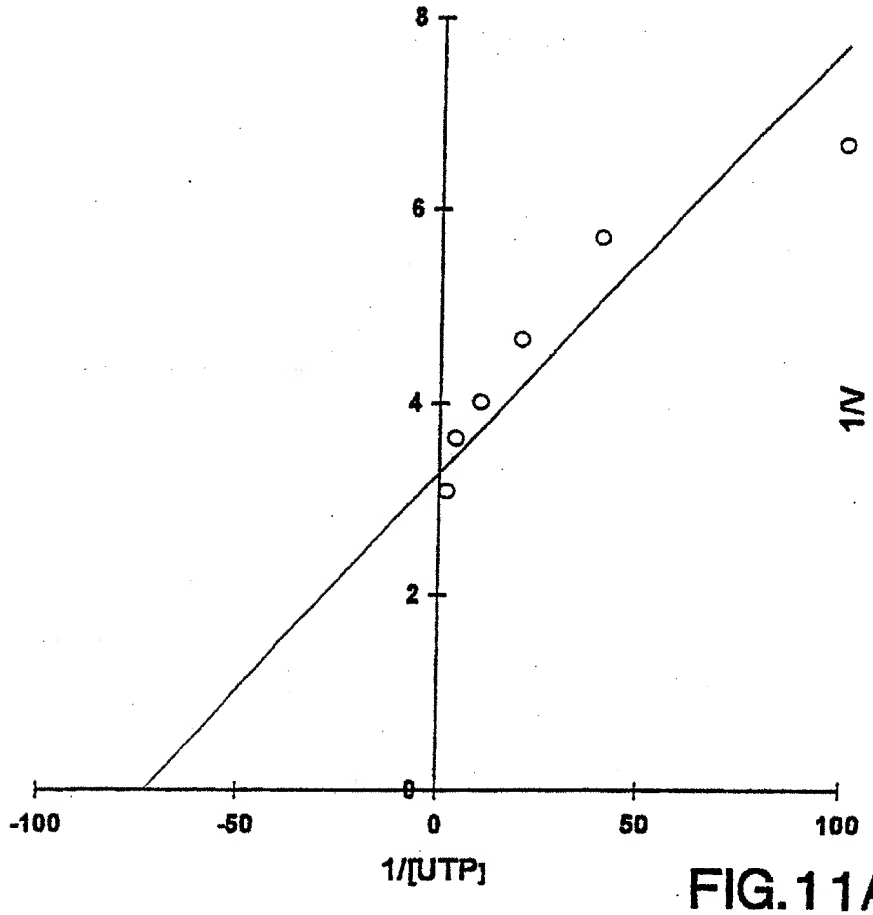

FIG.11A

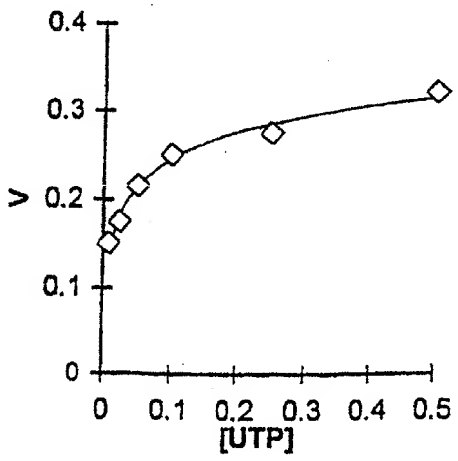

FIG.11B

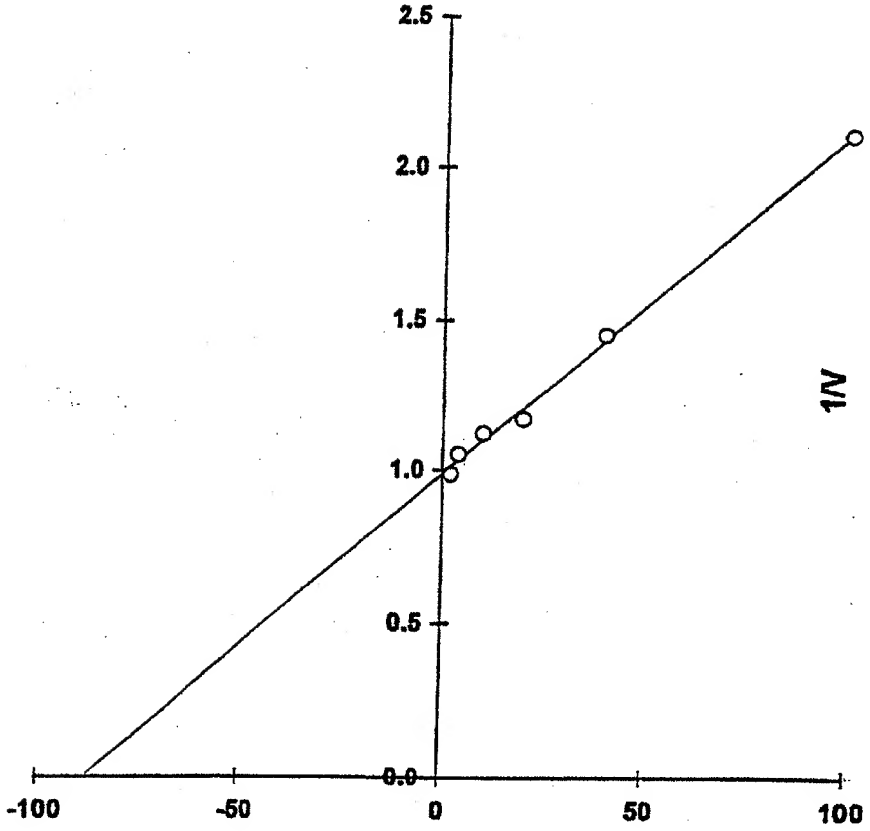

FIG.12A

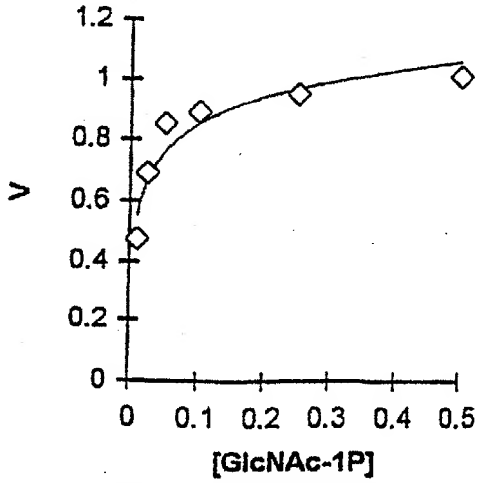

FIG.12B

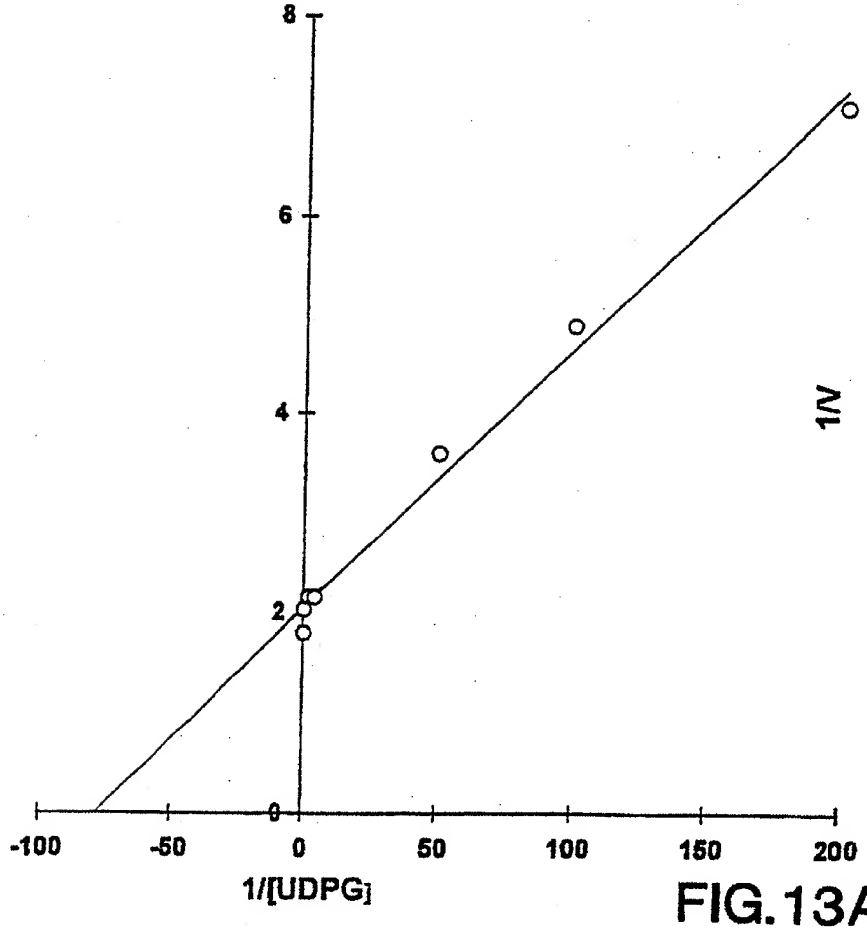

FIG.13A

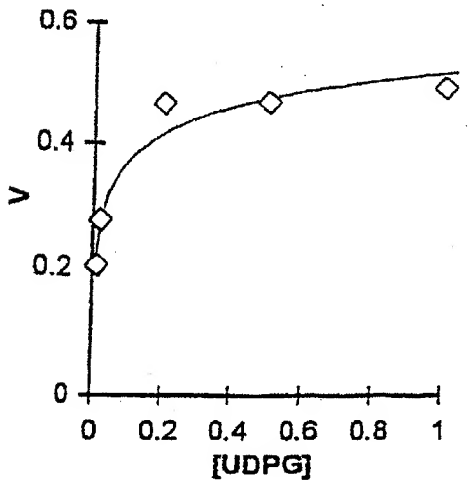

FIG.13B

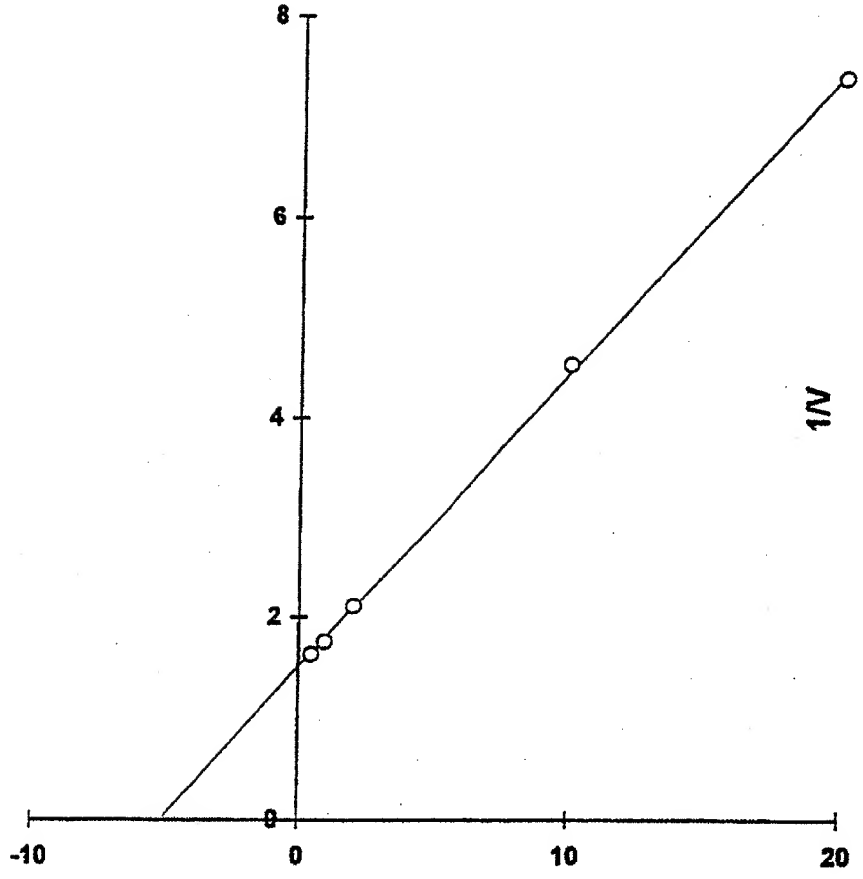

FIG. 14A

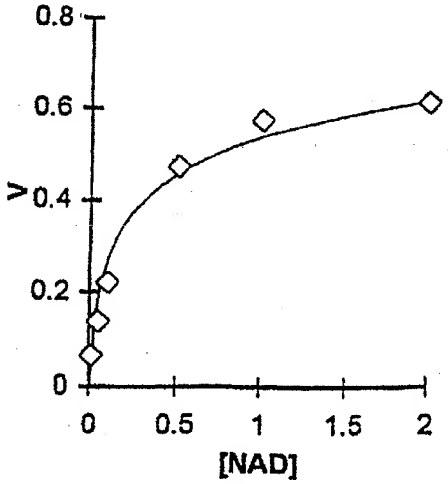

FIG. 14B

INTERNATIONAL SEARCH REPORT

Inte...tional application No.
PCT/US95/15600

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12P 19/04
US CL :435/101

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/101

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CA-ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A, 95/24497 (FIDIA ADVANCED BIOPOLYMERS S.R.L.) 14 September 1995, see entire document.	1-4
X -- Y	Biochemistry, Volume 33, Number 31, issued 09 August 1994, DeAngelis et al., "Immunochemical Confirmation of the Primary Structure of Streptococcal Hyaluronan Synthase and Synthesis of High Molecular Weight Product by the Recombinant Enzyme", pages 9033-9039, see Abstract.	1 ----- 2-4

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 01 MARCH 1996	Date of mailing of the international search report 25 MAR 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Sandra Saucier Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*